Author: Montoro, Pascal; See Chow, Keng; Li, Zhe; Phumichai, Thitaporn; Sales, Emma; Thulaseedharan, Arjunan; Thi Thuy Trang, Le
Year: 2011
Title: International Rubber Research and Development Board Biotechnology Group annual report 2010
Secondary Title: International Rubber Research and Development Board annual report for 2010
Place Published: Kuala Lumpur, Malysia
Publisher: IRRDB
Pages: 36-63
Type of Work: AP-Rapports d'activités
Short Title: International Rubber Research and Development Board Biotechnology Group annual report 2010
Original Publication: MontoroP_20130523; Mcl_20130524 (enregistrement "de guerre lasse")
Custom 5: 07-Rapports
Custom 6: A6-C3-13-Production non académique-Rapports d'Activités
Notes: Remarque: Ce rapport annuel de 34 pages se compose de 2 pages et demi de texte complétées par une liste bibliographique des travaux publiés le IRRDB Biotechnology Group au cours des années 2010 et 2011

Dépôt par MCL le 24 mai 2013

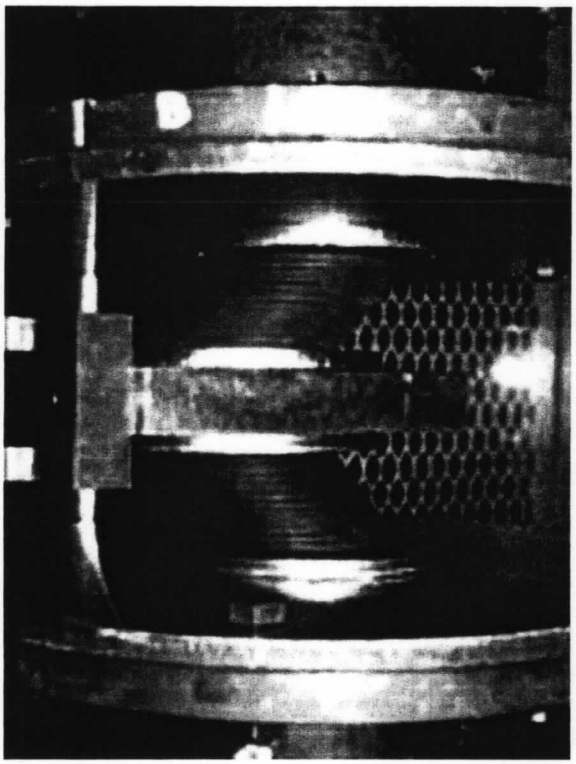
- ✓ Les copies de la couverture et de la page de titre se trouvent à la fin
- ✓ Pas de pdf disponible

V pas d'ISBN

In: *International Rubber Research and Development Board annual report for 2010.* Kuala Lumpur, Malaisia : IRRDB, 2011.

Annual Report

2010



IRRDB

The International Rubber Research and Development Board

International Rubber Research and Development Board

Annual Report for 2010

Secretariat: 260, Jalan Ampang, 50450 Kuala Lumpur

International Rubber Research and Development Board Biotechnology Group Annual Report 2010

By
Pascal Montoro, Keng See Chow, Zhe Li, Thitaporn Phumichai, Emma Sales, Arjunan Thulaseedharan, Le Thi Thuy Trang

ABSTRACT

The increasing demand for natural rubber calls for improved productivity and extension of rubber growing areas to non-traditional in most rubber growing countries. Biotechnologies play a role in breeding and also in understanding the various plant biological processes involved in plant development and defence against biotic and abiotic stresses. Research advances reported in 2010 confirm this trend.

Micropropagation and *in vitro* culture

Primary somatic embryogenesis is an efficient process to generate rejuvenated plant material. Usually based from anther or inner integument of immature seed, CATAS and RRII scientists obtained *in vitro* plants from root and leaf explants sources collected on juvenile plants, respectively. Long-term somatic embryogenesis is a more adapted way for large-scale production of *in vitro* plants, which consists of two main processes. First, the indirect secondary somatic embryogenesis combined with the cryopreservation was successfully developed on clone PB 260 and recently applied on clone RRIM 600 by CIRAD and Michelin. Second, the direct secondary somatic embryogenesis was established on a Chinese clone and 20 ha of field trial planted in the Guangdong Province in 2010.

Use of *in vitro* culture has proved tricky in *Hevea* breeding. After many years of research at RRII, the setting-up of pollen protoplast culture is still in progress with the aim of producing haploid tissue and subsequent homozygous plants. Besides, plants were regenerated and planted in field from 1 to 5-week-old rescued immature embryos and after polyembryony induction. These successes offer prospects for overcoming the incompatibility of certain intraspecific and interspecific recombinations.

Genetic modification

After Malaysia, India and France, Chinese scientists from CATAS obtained in 2010 transgenic plants using primary and secondary somatic embryogenesis. All these teams have provided genetic modifications for genes encoding proteins involved in the tolerance to abiotic stresses, the rubber biosynthesis and the production of recombinant pharmaceutical proteins. Overexpression of the transcription factor CBF1, osmotin and reactive oxygen species-scavenging enzymes (MnSOD, CuZnSOD) are expected to play a role in the defense against cold, drought and TPD, respectively. For instance, plants from a TPD-targeted CuZnSOD transgenic line displayed tolerance to water deficit at the juvenile

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stage of development. Finally, plants integrating new recombinant proteins such as the Human atrial natriuretic factor (hANF) and the Human protamine 1 (HP1) were regenerated and grown in nursery.

Following Malaysia, a first field trial with *Hevea* genetically modified is going to be implemented in India. Indeed, the Genetic Engineering Appraisal Committee considered on November 2010 the request of the Rubber Research Institute of India for permission to conduct limited-scale field trials of genetically modified rubber plants for the manganese superoxide dismutase gene [1].

Molecular physiology

Several studies have led to interesting results this year on latex regeneration (CATAS, CIRAD, RRII), tolerance to Tapping Panel Dryness (CATAS, CIRAD, RRII), wood quality (RRII) and tolerance to abiotic stresses (cold and drought) (CATAS, CIRAD, RRII). Two isoforms of cis-prenyltransferase (cis1 & cis2) are multigene families consisting of 4 and 2 genes, respectively (RRII). The invertase HbNIN2 and the sucrose transporter HbSUT3 were shown to be determining factors in the sucrose supply into latex cells (CATAS). Systematic analyses of the genes related to TPD suggest that the production and scavenging of reactive oxygen species (ROS), ubiquitin proteasome pathway, programmed cell death and rubber biosynthesis might play important roles in TPD (Li *et al.* 2010). Indian scientists also showed that peroxidase and several transcription factors are up-regulated in TPD trees. The cold-tolerant clone RRIM 600 was shown to accumulate transcripts from Lea 5, peroxidase, ethylene receptors and NAC transcription factors under cold treatment. Under drought stress, the later transcription factors seem to play also an important role.

Molecular phytopathology

Corynespora cassiicola leaf fall disease is intensively studied. Although isolates from Vietnam showed close genetic relation using rDNA ITS and ISSR markers (RRIV), some variation could be observed. Indeed, isolates were found to be deprived of the *cassiicolin* gene encoding the toxin thought to be responsible of the virulence (CIRAD, Michelin). Fungal endophytes partially inhibit the growth of *C. cassiicola* (CIRAD, Michelin). Besides, an anthocyanine glucosyltransferase and GRAS transcripts are accumulated under infection (RRII). Interestingly, a recombinant chitinase reduced the growth of *Corynespora* cultured in vitro (RRII). Finally, QTL mapping for the resistance to *Corynespora* implemented in Malaysia led to the identification of a major QTL (Cc1-1ian) and a minor (Cc1-2ian).

In Thailand, gene expression analysis was carried out after *Phytophtora palmivora* infection. This study revealed an induction of peroxidase, phenylalanine ammonia lyase and pathogenesis related-protein (3-glucanase, chitinase) genes.

Molecular genetics

Molecular genetic markers (ISSR, RAPD, SSR, SNP) are actively developed at CIRAD, RRII, RRIM, RRIT, RRIV and TARRC[1]. The microsatellite markers were successfully used in Vietnam and Philippines for the identification and authentication of *Hevea* clones. In Vietnam, genetic variability into a population of 250 genotypes (Amazonian, Wickham and hybrid resources) was studied using RAPD markers. The high variability observed in hybrids provided a source of material for selecting adapted *Hevea* clones to non-tra-

[1] Tun Abdul Razak Research Centre, Hertford, UK

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ditional rubber cultivation areas according to RRIV scientists.

Transcriptome and genome

To date, transcriptomes of clones PB 260, CATAS 7-33-97, CATAS 8-79, RRI 118 and RRIM 600 have been intensively sequenced by new generation of DNA sequencing (NGS) methods at CIRAD, CATAS, RRII and RRIM. Following these efforts for transcript sequencing, mostly from bark and latex RNA, functional annotation and characterization of genes have been easily carried out in several groups from CATAS, CIRAD, IRD, INRA/UBP[2], RRIM, MU[3] and RRII (cf. § molecular physiology). In addition to the transcriptome, non-coding RNAs called microRNAs were sequenced and their post-transcriptional role is under consideration at CIRAD.

The TARRC has worked in collaboration with the Genome Analysis Centre in UK to generate a high quality nuclear genome sequence from the latex-timber clone RRIM 928. Besides the nuclear genome sequencing, the sequence of the complete chloroplast genome was reported by the National Center for Genetic Engineering and Biotechnology in Thailand [2].

Conclusion

Control of various tools of biotechnology is beginning to allow applications in the area of molecular breeding and genetic modification. These applications are the result of extensive research that can be evaluated by the increasing number and quality of publications in journal with impact factor: ten in 2007, eleven in 2008, seventeen in 2009, seventeen in 2010 [3-19], and thirteen only for the first semester 2011 [2, 20-31]. This leads to workshops and seminars increasingly popular with researchers studying rubber. The recent Workshop on Biotechnology help in Kuala Lumpur on May 2011 concluded on needs for more scientific exchanges on micropropagation techniques, *Hevea* genome and molecular breeding, this latter requiring organizing a joint Workshop between the Biotechnology and the Breeding groups. Finally, training on Bioinformatics and Proteomics are also needed to improve skills of *Hevea* biotechnologists.

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[2]INRA/UBP: Institut National de Recherche Agronomique, University Blaize Pascal, Clermont-Ferrand, France

[3] MU: Mahidol University, Bangkok, Thailand

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CHINA - CATAS

The *Agrobacterium-mediated* genetic transformation system of rubber tree had been established in 2009 and 2010. In 2010, 11 transgenic plantlets through anther tissue culture short-term route were obtained verified by GUS staining, PCR detection, PCR product sequencing and Southern bloting analysis.

Using secondary somatic embryogenesis technique of rubber tree to proliferate transgenic somatic embryos, 464 transgenic regeneration plantlets from 5 independent transgenic embryo lines had been obtained (*uid*A and *npt*-II as exogenous genes). Among them 334 plantlets were transplanted on sand bed in nursery garden. 248 plantlets had survived.

Cold-induced transcription activator CBF1 gene had been transformed into the embryogenic calli from the anthers of *Hevea brasiliensis*. 44 putative transgenic regeneration plantlets had been obtained which had passed GUS staining, PCR detection and PCR product sequencing. Among them 34 plantlets survived in the nursery garden.

Somatic embryogenesis and plant regeneration using roots as explants in *Hevea* had been studied. From the *in vitro* axenic roots of rubber tree, embryogenic calli, somatic embryos, and more than 100 regeneration plantlets have been obtained.

Based on the established direct secondary somatic embryogenesis and plant regeneration technique, self-root juvenile test tube plantlets had been produced on a large scale. Nearly ten thousand plantlets were provided to Guangdong Province and planted. The testing cultivation area reached more than 20 hectares.

Several molecular marks related to the production of rubber tree had been discovered. It would help to select the parent plants for hybridization.

Through analyzing the regulatory pathways which TPD related genes participat-

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ing in, 7 possible key pathways were proposed. These results had been published on BMC Plant Biology.

The sucrose transportation protein key gene *HbSUT3* determining sucrose provision and influencing rubber production in laticifer had been identified. Two invertase genes participating in the utilization of sucrose in laticifer (*HbNIN1* and *HbNIN2*) had been cloned. It was preliminarily determined that *HbNIN2* was a key invertase gene that determined the utilization of sucrose in laticifer in normal tapping rubber tree. Six small G protein genes had been cloned (4 *Rab* and 2 *Arf*), the studies on their expression and regulation characters showed that they were tissue specifically expressed. 5 members in MYC transcription factor family and 8 members in JAZ family had been cloned from laticifer cells. It had been verified that MYC were transcription factors of *HbJAZ1 REF SRPP*.

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FRANCE - CIRAD

Establishment and cryopreservation of embryogenic friable callus lines for the clone RRIM 600

P. Besrest; F. Martin; S. Gaurel; F. Dessailly; C. Ledoux; L. Lardet; P. Montoro; F. Granet CIRAD and Michelin have kept friable callus lines by cryopreservation from several wild-type clones (PB 260, RRIM 703, PB 217, IRCA 109) and after genetic modification. Line CI07060 from clone PB260 is the most embryogenic one (average of 23 plantlets/g callus). *In vitro* plants from clone PB 260 are evaluated in field trial in Nigeria. In 2010, 18 new lines from clone RRIM 600 have been cryopreserved and their embryogenic potential is currently analysed.

Variation in GUS activity in vegetatively propagated *Hevea brasiliensis* transgenic plants

L. Lardet; J. Leclercq; E. Bénistan; F. Dessailly; G. Oliver; F. Martin; P. Montoro Fluorimetric GUS activity was efficiently used to assess the variation of transgene expression in *Hevea* transgenic *in vitro* plants and their subsequent budded sub-lines. The transgenic plant propagation method influenced GUS activity, which was not predictable in budded plants from the mother *in vitro* plant data. Regeneration of plants by somatic embryogenesis remains an effective way of propagating transgenic lines for functional analysis of genes in confined experiments. For large-scale trials, budding remains the most efficient rubber tree propagation system. The slight decrease in GUS activity in budded plants compared with *in vitro* plants does not appear to be detrimental and, in any case, *in vivo* characterization of plant material will be required for applications using genes of agronomic interest.

Characterization of CuZnSOD over-expressing transgenic lines

J. Leclercq; F. Martin; C. Sanier; A. Clément-Vidal; D. Fabre; G. Oliver; L. Lardet; A. Ayar; M. Peyramard and P. Montoro, Transgenic plant lines over-expressing a *Hevea brasiliensis* cytosolic *HbCuZnSOD* gene were successfully established and regenerated. Over-expression of the *HbCuZnSOD* gene was related to an increase in SOD activity in plant leaves. *HbCuZnSOD* gene over-expression impacted negatively somatic embryogenesis and allowed the production of two fast growing plantlets. The water deficit tolerance of these two *HbCuZnSOD* over-expressing lines was evaluated. The physiological parameters of transgenic plantlets subjected to a water deficit suggested that plants from line

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TS4T8An displayed lower stomatal conductance and a higher proline content. Over-expression of the *HbCuZnSOD* gene and activation of all ROS-scavenging enzymes also suggested that protection against ROS is more efficient in TS4T8An transgenic line.

Identification of conserved miRNA and their putative targets related to the control of redox status during plant development and abiotic stress in *Hevea brasiliensis*.

V. Gebelin; X. Argout; M. Rio; W. Engchuan; M. Ruiz; P. Montoro and J. Leclercq Vitroplants and budded plants were subjected to various abiotic stresses (cold, salinity, water excess and deficit, light excess, hormonal treatment). Micro RNAs were cloned and sequenced with the Solexa technology. The unique small RNA sequences were mapped against the PMRD database. From 670,645 unique sequences obtained (17-38 nt), 18,430 matched perfectly or near perfectly to known miRNAs in other species. The accessions were classified into 61 miRNA families among which 12 are predominant with more than 1,000 reads. Identification of miRNAs precursors were performed by using LeARN pipeline with the PB260 sequences obtained from several organs with the 454 technology. Nine miRNAs family mapped against sequences which displayed a stem-loop structure. Among them, 2 or 3 genes were identified. Putative miRNAs targets were detected by screening the *Hevea* EST database. Among them, known targets were identified. Interestingly, *Hevea* specific targets were also identified, five described for their differential expression between healthy and TPD tree, one involved in the cyanogenic potential and two in rubber biosynthesis and stability.

Identification and characterization of the AP2/ERF superfamily genes in *Hevea brasiliensis*

C. Duan; X. Argout; V. Gébelin; M. Summo; J.-F. Dufayard; J. Leclercq; M. Rio; A. Champion; P. Montoro A total of 153 putative AP2/ERF superfamily genes were identified in a transcript sequence database in *Hevea brasiliensis* clone PB 260 thanks to a phylogenetic analysis of the AP2/ERF domain. The alignments indicated that 3 clusters correspond to the AP2, ERF and RAV families. Twelve subclusters have been identified for the ERF family. Representative amino acid sequences of the AP2/ERF domain were selected and aligned for each family and each groups of the ERF family. Sixty-six tissue-specific genes were identified among the 153 AP2/ERF genes by comparison of their presence in the various transcript sequence libraries.

Characterization of the *cassiicolin* gene and analysis of its diversity among *C. cassiicola* populations

Marine Déon Yanice Bourré; Stéphanie Gimenez; Ricardo Oliveira; Shuhada Shuib; Daniel Bieysse; Angélique Berger; Frédéric de Lamotte; Véronique Roussel; François Bonnot; Gérald Oliver; Jérôme Franchel; Marc Seguin; Thierry Leroy; Patricia Roeckel-Drevet; Valérie Pujade-Renaud A collection of *Corynespora cassiicola* isolates of various geographical origins and from various hosts was screened for the detection of the cassiicolin gene. This revealed that some isolates deprived of *cassiicolin* gene could nevertheless be moderately virulent on rubber tree suggesting the existence of other disease effectors (or highly divergent forms of *cassiicolin*). Several isoforms of the cassiicolin gene were identified. Their expression *in planta* is being analyzed.

Search for antagonists of *C. cassiicola* among rubber tree endophytes

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Marine Déon, M.; Romina Gazis, R.; Ana Scomparin, A.; Thierry Leroy; Patricia Roeckel-Drevet; Priscilla Chaverri; Valérie Pujade-Renaud The antagonistic potential of fungal endophytes of rubber tree against a pathogenic *Corynespora cassiicola* isolate was investigated *in vitro*. Most endophyte species tested could partially inhibit the growth of *C. cassiicola* (15 to 30 % inhibition) but only a few had the capacity to develop and sporulate on top of the *C. cassiicola* colony (mycoparasitism). These species should then be tested in planta to evaluate their potential for the biocontrol of *C. cassiicola* in rubber tree.

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INDIA – RUBBER RESEARCH INSTITUTE OF INDIA

1. *In vitro* studies

Sushama Kumari, S.; Kala, R.G.; Kumari Jayasree, P.; Jayasree, R.; Rekha, K.; Sobha, S. and *Thulaseedharan, A.*

a. Somatic embryogenesis

Extensive experiments were carried out to refine the somatic embryogenesis and plant regeneration pathway developed earlier from leaf explants collected from glass house grown bud - grafted plants of *Hevea* clone RRII 105. Embryo induction was tried from embryogenic callus obtained from proliferated friable callus obtained from newly initiated leaf cultures. Experiments were also carried out to increase the rate of embryo induction from the proliferated embryogenic calli. The concentration of calcium nitrate, phytohormones and phytagel were varied during further subcultures according to the texture of the callus to be subcultured. Accordingly the hormone combination and concentration were changed with inclusion of ABA (0.2 mg/l) along with polyethylene glycol (5.0 g/l) and increasing the concentration of phytagel. Rate of embryogenesis could be increased with 80% embryo induction after 3 months culture.

Leaf explants collected from physiologically juvenile source plants gave good response to *in vitro* culture. Experiments were done to find molecular factors if any related to tissue juvenility and *in vitro* response. Work was done to identify the presence of genes controlling phase changes in plants. The gene coding for chlorophyll a/b binding protein gene (CAB) was successfully PCR amplified, cloned and sequenced. RT PCR with cDNA obtained from tissues with different levels maturity showed that the gene is differentially expressed with more expression in juvenile tissues.

Experiments were continued for plant regeneration through somatic embryogenesis form immature anther explants. Regenerated plantlets were hardened and field planted. Attempts were also continued to develop a regeneration system from unfertilized ovules and to use the embryogenic callus as a target tissue for genetic transformation experiments. The callus obtained from the micropylar end was isolated and cultured for proliferation. The proliferation of the callus was achieved in MS basal medium supplemented with reduced levels of growth regulators.

b. Embryo rescue and induction of polyembryony

Refinement experiments by manipulating the media components, for the rescue of immature embryos (1-5 week) were continued. More promising results were obtained when kinetin was replaced with zeatin in combination with GA_3. A steady increase in the embryo recovery was observed with increase in concentration of zeatin up to 3.0 mg l^{-1}. Plants were recovered and

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established in the field. Experiments for the induction of polyembryony were also continued. Multiple seedlings of single origin were developed, hardened and field planted. RAPD and microsatellite analysis could prove the genetic uniformity as well as zygotic origin.

c. Pollen protoplast culture

The lengthy and tedious process of creating a pure line in an open pollinated, highly heterozygous tree species like *Hevea* can be overcome by regenerating haploid tissues after culturing pollen grains and unpollinated ovules and subsequent diploidisation of the regenerated tissue. As an initial step towards this end, the effect of different levels of enzymes and osmotica on pollen protoplast isolation was experimented. The yield of protoplasts was highest when a mixture of 0.5% cellulase and 0.05% pectolyase was used in the presence of 0.6M mannitol and 0.3% sorbitol. For purification of the released protoplasts, two methods *viz.* density gradient centrifugation and sieving were attempted. Density gradient centrifugation was not effective for separation of the pollen protoplasts whereas the protoplasts could be partially purified by sieving through a 64 µm mesh. These protoplasts were cultured in the nutrient medium.

2. Genetic transformation

Sushama Kumari, S.; Kumari Jayasree, P.; Kala, R.G.; Jayasree, R.; Rekha, K.; Sobha, S. and Thulaseedharan, A.

Genetic transformation experiments were continued to develop transgenic plants with increased tolerance to abiotic stresses, tapping panel dryness and higher latex yield using the genes coding for superoxide dismutase under the control of CaMV 35S and FMV 34S promoters, sorbitol-6-phosphate dehydrogenase, isopentenyl transferase, hmgr1 and osmotin protein. Extensive optimization experiments were carried out to improve the transformation efficiency and regeneration of transgenic tissues. New *Agrobacterium* infections were performed using different explants *viz;* embryogenic callus of immature anther, ovule and polyembryony derived callus.

Experiments were done to improve the transgenic callus proliferation rate and texture, which in turn improve the transgenic plant regeneration efficiency in SOD transgenic lines obtained from immature anther derived callus. Callus proliferation was favored by the addition of lower levels of 2,4-D and NAA, while higher concentrations of 2,4-D induced hard and compact callus. A callus proliferation frequency of around 80% was obtained with a growth regulator concentration of 0.80 mgl^{-1} 2,4-D [1]and 0.4 mgl^{-1} NAA. The transgenic cell lines obtained earlier from the *Agrobacterium* infected immature anther derived callus were proliferated and sub cultured for embryogenesis in medium supplemented with PEG and ABA and transgenic embryos were induced at a frequency of 75%. Transgenic plants were regenerated and are being hardened.

Northern blot analysis was repeated to assess the over expression of MnSOD gene in the transgenic plants L1 & L2 integrated with MnSOD gene developed earlier. Transgenic plant L1

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showed a higher MnSOD transcript level than in L2, whereas the expression pattern was very low in the bud-grafted control RRII 105 and in the non-transgenic somatic plant of the same clone. The transgenic plants L1 & L2 were multiplied by bud grafting along with control plants for initiating field trial.

Genetic transformation experiments were carried out using different explants for the integration of osmotin gene following a modified procedure. Transformation frequency could be enhanced by adopting liquid co-culture. Out of the 70 transgenic cell lines obtained, 50 could be proliferated in MS medium supplemented with 1.0 mgl^{-1} NAA along with 1.5 mgl^{-1} 2,4-D. Amplification of the gene insert was observed in randomly selected lines tested. Experiments were performed by manipulating the media components for embryo induction. Embryo induction frequency could be enhanced from 36-50% when Kin and ABA were supplemented along with already developed media. A growth regulator combination of 0.3 mgl^{-1} Kin, 0.1mgl^{-1} ABA and 2 mgl^{-1} NAA along with 10 gl^{-1} of mannitol was found to be ideal for embryo induction. Embryos were matured (15%) and a few has been germinated (2%) and are in the process of hardening.

Attempts were also continued to develop an *Agrobacterium* mediated genetic transformation and plant regeneration protocol using leaf explants as the target tissue for *Agrobacterium* infection. Experiments were carried out to enhance transformation frequency and improve the texture of emerging transgenic lines by modifying the infection medium. Positive results could be obtained by inclusion of anti oxidants and surfactants. The transgenic embryogenic callus obtained earlier incorporated with MnSOD, isopentenyl transferase *(ipt)* and TB antigen genes were further subcultured for embryo induction and plant regeneration. Embryo induction and maturation has been obtained from transgenic lines incorporated with *ipt*, MnSOD gene and TB antigen genes. A few plantlets were also regenerated incorporated with *ipt* and MnSOD genes.

Work was continued to develop transgenic rubber plants integrated with HMGR 1 gene under the control of super promoter, using the embryogenic callus derived from the polyembryonic tissue. Transgenic plantlets were produced from these embryos. The frequency of plant germination was 20%. The plantlets were successfully acclimatized and maintained in polybags. This is the first report on the regeneration of transgenic plants integrated with the *HMGR1* gene in *Hevea*.

3. Molecular mechanism of abnormal leaf fall disease tolerance

Thulaseedharan, A.

The earlier studies on isolation and characterization of β-1,3-glucanase gene involved in abnormal leaf fall disease tolerance in *Hevea* lead to the identification of five isoforms of β-1,3-glucanase gene in a single *Hevea* clone RRII 105. These different gene isoforms were controlled by separate promoters. Attempts were continued to develop promoter: GUS fusion binary vectors and measure the GUS expression in the heterologous system through transgenic approaches. The promoter fragments have been cloned in pCAMBIA 1381 Z TDNA vector upstream to the

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GUS A gene. All the five promoters identified were cloned in to the binary vector with and with out deletion. Total eleven binary vectors with different sizes were developed. After confirming the presence of the inserts of different lengths in the above mentioned binary vectors, the constructs have been transformed into the competent Agrobacterium strain EHA 105 and selected in LB agar plates containing 50 µg/ml kanamycin and 20 µg/ml rifampicin. The positive transformants has been confirmed through colony PCR for the presence of vector constructs with the insert, GUS and *hpt* II (hygromycin resistance gene for plant selection). Later these positively transformed *Agrobacterium* have been used to transform tobacco.

4. Characterization of *cis*-prenyltransferase gene isoforms from *Hevea*
Thulaseedharan, A.

The relative copy number of *cis*-prenyltransferase gene isoforms 1 & 2 genes were studied through ΔΔCT method in a high yielding *Hevea* clone, RRII 105 and a low yielding clone RRII 33. A C_T difference of "1" was observed between *cis* - 1 & 2 gene isoforms. *Cis*-2 gene has shown a high CT value indicating the copy number of *cis*-2 is only half of *cis*- 1. The absolute copy number of *cis*-1 and 2 gene isoforms were studied through standard curve method. Plasmid carrying cloned *cis*-prenyltransferase1 gene was used as the standard. *Cis*1 and *cis*2 were amplified together in the standard curve experiment with plasmid and the copy number of *cis*1 was found to be double of *cis* 2. From the results it is calculated that the haploid genome of *Hevea* contain 4 copies of *cis*-1 and 2 copies of *cis*-2.

5. DNA markers & genetic linkage map
T. Saha, Bini K, M. Ravindran

Microsatellite markers were generated from an enriched genomic library for dinucleotide repeats and successfully used in the characterization of cultivated popular Wickham clones as well as wild *Hevea* germplasm accessions.

Genomic DNA sequences of HMGR gene (2.33 kb) from five *Hevea* clones were analyzed for SNP detection. Ten SNPs and one indel were identified. Two distinct haplotypes were predicted.

Marker segregation data is being continuously integrated into the mapping data to populate the linkage map of rubber. Total 215 marker loci were used to genotype 60 progenies along with their parents RRII 105 and RRII 118. In RRII 105, 72 loci were distributed in 18 linkage groups and 74 remained unlinked. Whereas 20 linkage groups were formed with 65 loci in RRII 118 and 72 loci were unlinked.

6. Genes involved in fungal disease resistance/tolerance
T. Saha, Bindu Roy , M. Ravindran

Analysis of RT-RGA13, one of the functional resistance gene analogue identified in rubber, showed over-expression in *Corynespora* infected rubber clones and therefore full-length cloning of the same was performed.

Sixty differentially expressed transcripts derived from *Corynespora* challenged leaf samples of RRII 105 were cloned and sequenced. Involvement of a set of unique transcripts was identified during disease establishment.

Over expression of the cDNA fragments of anthocyanidine glucosyl-

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transferase and GRAS from rubber due to *Corynespora* infection was confirmed through reverse northern analysis.

7. **Methylation of *Hevea* genome**
K.U. Thomas, T. Saha, M. Ravindran

Regulatory sequences of four major genes involved in the mevalonate pathway (rubber biosynthesis pathway) and one common defense related gene of three high yielding popular rubber clones grown at two different agro-climatic conditions were analyzed for the presence of methylation. Several significant variations in the methylation pattern at core DNA binding motifs within all the five genes were identified.

8. **EST-sequencing**
T. Saha, K.U. Thomas, M. Ravindran

In EST generation program, cDNA inserts were amplified from the clones of a bark cDNA libray of RRII 118 and sequenced. Sequence annotations were performed.

9. **Lignin biosynthesis genes for wood quality improvement**
T. Saha, K.U. Thomas, M. Ravindran

A full-length CCR gene involved in lignin biosynthesis was sub-cloned into pRSET-A vector for bacterial expression. Expression of CCR gene construct was achieved. A recombinant protein band (42 kD) was detected.

10. **Molecular basis of TPD**
Mohamed Sathik and Molly Thomas

a. Investigations on the molecular physiology of tapping panel dryness syndrome (TPD) in *Hevea brasiliensis:* Cloning and Characterization of TPD Responsive Genes by Subtractive Hybridization.

The EST sequences obtained through subtractive hybridization experiment were submitted in the dbEST database of GenBank and the accession numbers for the same have been obtained (GR305128 – GR305928). qPCR primers were designed and synthesized for endogenous control genes such as cyclophilin, ADP ribosylation factor, GAPDH, elongation factor, polyubiquitin, H1/H5, H3, ACBP and ferredoxin. Based on the primer efficiency (slope value) and Ct value, primers for cyclophilin, ADP ribosylation factor, GAPDH and polyubiquitin were selected for further analysis. Primers were standardized for their efficiency, primer usability and for assessing the expected threshold cycle value of each primer. Among the primers tested, the primers such as cyclophilin, GAPDH, ADP ribosylation factor and polyubiquitin gave good amplification in which GAPDH was found better for drought.

Bark samples were collected from TPD affected (late dripping trees, 10%, 25%, 50% and 75% TPD) and normal trees. cDNA synthesized from these samples were used as template for the qPCR study. Though cyclophilin and GAPDH genes were found suitable as endogenous control by '*M*' value (Vandesompele *et al.*, 2002), GAPDH was used as endogenous control for the qPCR analysis using Applied Biosystems' 7500 Real Time PCR System.

Out of the twenty genes analyzed, four genes were found highly up-

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regulated and one down-regulated remarkably in the initial stages of TPD trees. Peroxidase and Transcription factor MBF (tf MBF) showed significant level of up-regulation in the TPD trees. The significant up-regulation of peroxidase and tf MBF found in TPD trees indicates the possibility of increased levels of reactive oxygen species in TPD trees and the same was already proved by physiological experiments. NAC transcription factor (NAC tf), TPD 24 and HbDRT 5b were also up-regulated in TPD trees when compared to healthy trees. WRKY transcription factor (WRKY tf) showed increased levels of expression in the initial stages of TPD whereas CRT/DRE binding factor showed down regulation.

Tf MBF1 is a transcriptional co-activator that mediates transcriptional activation by bridging a sequence-specific activator and TATA-box–binding protein (TBP) (Li *et al.*, 1994). In *Arabidopsis,* MBF1c has been reported to be produced more in response to pathogen infection, salinity, drought, heat, hydrogen peroxide and plant hormones such as abscisic acid or salicylic acid (Rizhsky *et al.*, 2004b; Tsuda and Yamazaki, 2004). Tf MBF1 expression had also been shown to enhance the tolerance to heat and osmotic stress by partially activating, or perturbing the ethylene-response signal transduction pathway in transgenic plants (Suzuki *et al.*, 2005). Higher levels of expression of Tf MBF1 proteins in TPD trees indicate that this might be involved in triggering the transcription of stress alleviating genes against increased levels of ethylene stress which has been proven so in TPD trees (Krishnakumar *et al.*, 2006). The transcription of WRKY tf is strongly and rapidly triggered in response to wounding, pathogen infection or abiotic stresses in numerous plant species (Eulgem, 2000).

b. Involvement of Ethylene in *Hevea* - Rubber Biosynthesis and Tapping Panel Dryness.

A new set of primers were designed and synthesized. Coding region of β-cyanoalanine synthase and ACC Oxidase were PCR amplified and further cloned into the cloning vector. DNA sequencing has been carried out and arrangements were made to clone into the expression vector.

11. Environmental Physiology

Molly Thomas and Mohamed Sathik

a. Molecular studies on cold stress in *Hevea*

Attempts were made to investigate the molecular basis of cold tolerance in *Hevea* with special reference to identification of genes/regulatory factors associated with cold tolerance that could be employed for selection of genotypes suitable for low temperature prone regions of India.

Six months old plants (grown in polythene bags) of clones RRII 105 (cold susceptible) and RRIM 600 (cold tolerant) were acclimatized in a growth chamber for three days with a minimum temperature of 15°C during night (for 3 hours) and a gradual rise in the maximum temperature up to 25°C in the day time. Fourth day onwards, cold treatment was imposed at 8°C for 3 hours between 2:00 and 5:00 am

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followed by a gradual increase in the maximum temperature up to 16°C during the day time for five consecutive days mimicking ambient conditions during winter in NE India. The control plants were allowed to grow at stress free and ambient weather conditions of RRII. Cold induced inhibition of photosynthesis in the low temperature treated plants was confirmed by monitoring the CO_2 exchange rate using a portable photosynthesis system. Leaf sample collection, mRNA isolation, cDNA synthesis and quantitative PCR (qPCR) were performed as per the standard protocols. GAPDH was used as endogenous control in qPCR analysis. Out of the twenty genes attempted, Lea 5 protein and peroxidase were shown to be up-regulated in RRIM 600 when compared to RRII 105, indicating that these proteins might be contributing for cold tolerance in clone RRIM 600. ETR1, ETR2 and NAC transcription factor (DRT 5b and TPD 24) were also highly up-regulated in tolerant clone, RRIM 600.

LEA-type proteins have been reported to be expressed in response to water deficit resulting from desiccation, cold and osmotic stress in a wide range of plant species. The over expression of LEA proteins have been implicated to play a role in cellular protection during the stress (Vierling and Kimpel, 1992). NAC (NAM, ATAF, and CUC) is a plant specific transcription factor family with diverse roles in development and stress regulation. Reports suggest that NAC tf are induced by drought, salinity, cold, wounding and abscisic acid (ABA) treatment (Honghong Hu, 2008).

b. Molecular studies on drought stress in *Hevea*

This study was attempted to understand the molecular mechanisms of drought stress tolerance and to study the response of various *Hevea* clones to drought treatment. For this purpose, clones such as RRII 105 (susceptible), RRIM 600 (tolerant), RRII 208 (tolerant) and RRII 430 (tolerant) were selected. One year old polybag plants were raised in RRS, Dapchari (a drought prone area in central India) and were imposed with drought for fifteen days in natural condition during the summer period of 2010. After 15 days of drought treatment, drought inhibition of photosynthesis and the stress impact was assessed in the leaves by using a portable photosynthesis system. After confirming the impact of stress by gas exchange parameters, leaf samples were collected. mRNA was isolated and cDNA was synthesized for quantitative expression studies. Seven genes were selected for the quantitative expression study. GAPDH was used as endogenous control for qPCR analysis.

For each treatment, three biological replications were included in the qPCR analysis. Statistical analysis was performed with the relative quantification data using ANOVA. The ratio with a P-value <0.05 was adopted as significant for down- or up-regulation.

Among the seven genes studied, NAC tf (DRT 5b and TPD 24) was found up-regulated in drought exposed plants of RRIM 600, RRII 430 and RRII 208 when compared to

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RRII 105 control. This significant level of up regulation in drought tolerant clones shows close association of NAC tf with drought stress. Transcripts of TPD 27 was found abundant only in drought treated sample of RRII 430, whereas, there is no much change between the control and treated samples of RRII 430 and RRIM 600. All the six genes except DRT 82b were found significantly up-regulated in drought treated sample of the clone RRII 430 compared to RRII 105 control plants.

12. Construction of an expression vector for over-expression of chitinase in endophytes of *Hevea brasiliensis.*
Mohamed Sathik., Shaji Philip and Molly Thomas

Expression studies of transformed endophytic *B.subtilis*/pHT43/ chitinase gene was conducted and confirmed the non-specific amplification of 1kb band from pHT43 expression secretion vector. Vector specific primers were designed and synthesized again to confirm the cloning of chitinase gene in the pHT43 vector. Lac I repressor was removed from pHT43 vector and the ends were filled, ligated and transformed in to *E.coli* cells and the clones were confirmed by PCR and restriction digestion analysis. The clones were sequenced and selected based on the sequence for further cloning into the pHT 43 vector. Arrangements are being made to transform the vector into modified cells which can accommodate the pHT43 vector with chitinase and facilitate the expression of the cloned coding sequence of chitinase.

13. Cloning and production of HMG-CoA protein of *Hevea* for Immunoassay analysis
Mohamed Sathik and Krishnakumar, R.

As a measure to analyze if HMG CoA is a rate limiting step in the rubber biosynthesis pathway, this project was initiated. The main mode of approach here is to raise antiserum of this particular protein and use it to screen high yielding clones. For this purpose, the coding region of this particular protein need to be PCR amplified and cloned into expression vector for protein synthesis. In this cotext, primers have been designed and synthesized. PCR amplified products were cloned and sequenced. The results are being analyzed.

14. Molecular Mechanism of host pathogen interaction in *Corenyspora* leaf diseaseof *Hevea:* Expression of chitinase gene, purification and antifungal activity of recombinant protein.
Shaji Philip

The 978 bp chitinase gene was PCR amplified from the *Hevea* clone GT1 using chitinase specific primers flanked with restriction sites (BamHI in the forward primer and NotI in the reverse primer). The expression system pET 32a+ (Novagen, Germany) was tried for recombinant protein expression. The strain BL 21 (DE 3)/ BL 21(DE 3) were used for the transformation and expression of target chitinase gene. The expression targeted protein was induced by addition of IPTG to the growing culture. The cells were harvested at 1.5 hrs and 3 hrs. The samples were analyzed through SDS-PAGE. The control vector without insert was also analyzed along with induced and uninduced samples. In pET vector, a very high induction of the fusion protein (Trx+ Target gene) was detected. A ~46 kD band was observed in all induced samples.

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The fusion protein expressed in pET vector was effectively purified in milligram quantities with His Bind purification kit. The purified protein was tested on 10% SDS PAGE. The antifungal activity of purified protein was assayed on PDA plates which are inoculated with a growing culture of *Corenyspora* using filter paper disc method. The recombinant protein inhibited the fungal growth. The recombinant protein was send for raising polyclonal antibodies and development of an ELISA based detection system.

15. Development of SCAR marker for Phytophthora tolerance from *Hevea* clones

Shaji Philip

Seven *Hevea* clones *viz;* Tjir1, GL1, RRII 105, HB, RRIC 100, RRIM 600 RRII 430 were selected for the present study. Among the clones, GL1, RRII 105, HB are *Phytophthora* tolerant lines and Tjir1, RRIC 100 and RRIM 600 are susceptible clones. DNA was isolated form clones by CTAB method and RAPD analysis was carried with 120 random primers. Out of the 120 primers screened, OPA 4 and OPF 13 primers showed polymorphic banding pattern with susceptible parent. The amplifications were repeated thrice and the reproducible bands were sent for sequencing. RAPD analysis of isolated DNA with more primers is in progress.

16. Molecular approaches for inducing ISR/SAR in *Hevea* clones using beneficial microbes/chemicals

Shaji Philip

Twenty one endobacterial isolates form the culture collection of RRII was selected for the antagonist study. The selected endophytes were tested for the ability to inhibit the growth of pathogen by dual culture test. Based on the dual culture studies, the bacteria 600 ptj. Jpte and Gpta were selected for further studies. The antagonist bacteria belong to *Pseudomonas* and *Bacillus sp.*

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MALAYSIA

Rubber Research Institute of Malaysia - Malaysian Rubber Board (MRB)

LATEX PHYSIOLOGY

Elucidation of Biochemical Mechanism of Latex Vessel Plugging in *Hevea brasiliensis*

Latex vessel plugging happens when latex is destabilized as it exudes from the rubber tree due to the formation of plugs at the vessels and consequently the latex flow stopped. The main objective of this study is to elucidate the involvement of latex proteins mainly in B serum and its role in latex vessel plugging using 2 DE methods. Proteome comparison between B serum and *in vitro* destabilisation has identified proteins suspected in latex destabilisation and may also involve in latex vessel plugging. Further analysis is required to verify the proteins' function and their involvement in latex destabilisation.

LATEX NUTRACEUTICAL

Extraction of Value-Added Minor Components from Rubber Processing Waste using Supercritical Fluid Extraction (SFE)

The aim of this study was to extract and identify the value-added minor components mainly lipids from the rubber waste of DPNR (De-Proteinised Natural Rubber) processing, preventing its loss in the effluent. The extraction was carried out by using liquid-liquid extraction method and Countercurrent Supercritical Fluid Extraction (CC-SFE) method and analyzed quantitatively by HPLC (High Performance Liquid Chromatography). It is expected that the DPNR serum will have high lipids content that contains the value-added minor components. A new process based on the use of countercurrent supercritical fluid extraction (CC-SFE) for extracting value-added minor components from DPNR serum has been introduced. It could allow the advantages of SFE method over classical extraction method (liquid-liquid extraction) with short times, low cost and no toxic fluids used as extractants. Liquid-liquid extraction and SFE methods revealed similar result. The analysis shows that tocotrienols isomers were presented in the DPNR serum extract.

GENETIC TRANSFORMATION

Enhanced recombinant pharmaceutical production from transgenic *Hevea* by the use of latex-specific promoters

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Genetic engineering is an interesting tool for the production of recombinant proteins of interest and improvement of the agronomic traits of *Hevea brasiliensis*. The genetic engineering program requires the use of efficient promoters, well adapted to the *Hevea* plant and the application aimed, in order to optimize the expression of the transgene at the specified tissue. For the production of recombinant proteins in the latex of rubber trees, a strong latex specific promoter is the preferred choice. One such candidate is the promoter for the latex protein; hevein.

Genetic transformation experiments were performed to evaluate the hevein promoter fragments of varying lengths (HevP1 - 0.35 kb, HevP2 - 0.45 kb, HevP3 - 0.73 kb) for induction of *Human atrial natriuretic factor* (hANF) and *Human Protamine 1 (HP1)* gene expression. Human atrial natriuretic factor (hANF) is a potent vasoactive cardiac peptide-hormone while HP1 is a therapeutic protein which is commonly used to neutralize the anticoagulant effects of heparin during cardiovascular surgery. The hANF or HP1 cDNA was cloned downstream to the promoter/upstream regulating sequences of the hevein gene (that encodes for the most abundant soluble protein in the latex cytosol) in pGPTV-Kan expression vector. Transformed plantlets regenerated from earlier co-cultivation were maintained in the transgenic netted house (nursery). PCR amplifications performed on leaf genomic DNA revealed the presence of hANF, HP1, nptII and the respective hevein promoter fragments in all the tested plantlets.

RUBBER GENOMICS AND BIOINFORMATICS

Latex Transcriptomics

As a result of combining Sanger and second generation DNA sequencing platforms, a non-redundant collection of more than 20,000 latex gene transcripts is available. The extent of gene coverage in this dataset was confirmed by analysis of known rubber open reading frames and size length distribution. Functional annotation of these latex gene transcript sequences was performed by BLAST2GO analysis. This resulted in their classification into the GO (Gene Ontology) categories: molecular function, cellular component and biological process. In the molecular function category, a major proportion of transcripts (75.25%) encoded gene products with binding and catalytic activities. The cellular component category showed that more than half of the transcripts (59.42%) were associated with structural and constituent parts of the cell unit. In the biological process category, 41% of transcripts displayed functions in cellular (21.70%) and metabolic (19.30%) processes. GO classification has reinforced the cellular nature of the laticifer.

Rubber Genome Sequencing

A *Hevea brasiliensis* latex-timber clone, from the RRIM 928 clone was selected for whole genome sequencing. The genome sequencing project was led by the Tun Abdul Razak Research Centre (TARRC) in Hertford, UK in collaboration with the Genome Analysis Centre (TGAC), Norwich, UK. Sequencing was performed using the Illumina and 454 sequencing platforms. Both sets of sequence reads were assembled using software developed by TGAC. Transcriptome sequences from leaf and latex of the same clone were generated at MRB Sungai Buloh to assist in genome annotation. In October 2010, a high quality genome sequence was announced in conjuction with the MRB Technology Day.

MOLECULAR PHYTOPATHOLOGY

CONSTRUCTION OF GENETIC LINKAGE MAPS FOR TWO *HEVEA* FAMI-

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LIES: PB 5/51 X IAN 873 AND RRIM 937 X RRIM 600

Two mapping populations were used in the genetic linkage mapping project *i.e.* PB 5/51 × IAN 873 and RRIM 937 x RRIM 600 using Amplified Fragment Length Polymorphic (AFLP) markers. Six skeleton maps were generated, one for each clone and consensus map for each cross. The details of the map are summarized in *Tables 1 and 2.*

for linkage group 1, *1* is the first QTL detected, *ian* for IAN] was located on Group 1 of IAN 873, which explained 10.1% of the phenotypic variation and a minor QTL (*Cc1-2ian*) [*Cc* for *C. cassiicola*,1 for linkage group 1, *2* is the second QTL detected, *ian* for IAN] was located on the same linkage group, explaining 5.2% of the phenotypic variation. IAN 873 is known to be susceptible to *C. cassiicola*, thus it can be assumed

Table 1: Description of the map data for PB 5/51, IAN 873 and consensus map PB 5/51 x IAN 873 PB 5/51 IAN 873 Consensus Map

	5/51	873	nsensus Map
Number of markers	277	174	214
Number of groups	19	17	18
Number of markers with 1:1 ratio	60	86	-
Number of markers with 3:1 ratio	167	88	75
Number of linked markers	63	60	75
Number of unlinked markers	214	114	139
Map lengtha	276.8	272.3	241.9
Marker density (cM)	4.4	4.5	3.2

aCorresponds to the sum of linkage group sizes, established with JoinMap ver 4.0.

Table 2: Description of the map data for RRIM 937, RRIM 600 and consensus map RRIM 937 x RRIM 600

	RRIM 937	RRIM 600	Consensus Map
Number of markers	125	123	122
Number of groups	15	12	11
Number of markers with 1:1 ratio	33	21	
Number of markers with 3:1 ratio	92	102	122
Number of linked markers	38	10	34
Number of unlinked markers	87	113	88
Map lengtha	180.3	36.2	241.9
Marker density (cM)	4.7	11.3	7.1

aCorresponds to the sum of linkage group sizes, established with JoinMap ver 4.0.

QUANTITATIVE TRAIT LOCI (QTL) MAPPING FOR RESISTANCE/SUSCEPTIBILITY AGAINST *C. CASSIICOLA*

Two putative QTLs were detected on the genetic map of IAN 873.

Using the interval mapping approach, a major QTL (*Cc1*-1ian) [*Cc* for *C. cassiicola*,1

that these QTLs are associated to susceptibility to the fungus *(Fig. 1A).*

The subsequent multiple-QTL model (MQM) analysis was also conducted and did not reveal any new QTLs on any other linkage groups. MQM analysis was able to pin-point the positions of the QTLs closer to markers P2E2M13205 and P2E2M13198/P2E2 M13220 for *Cc1-1ian*

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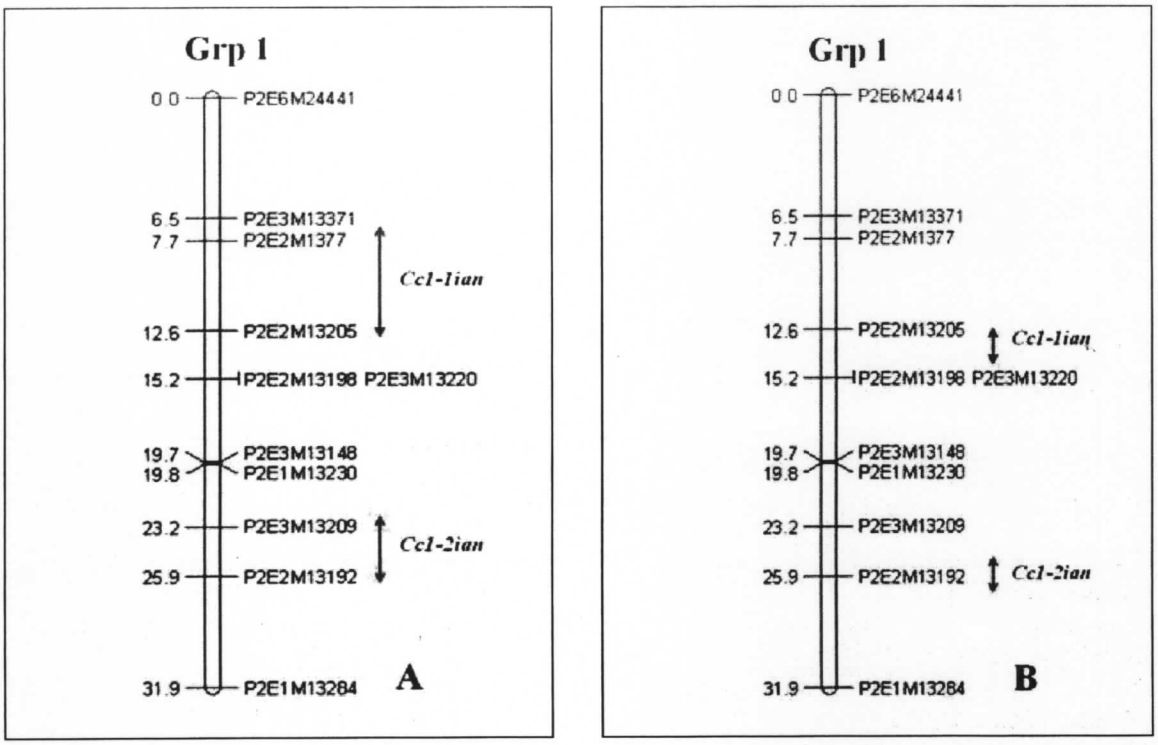

Fig 1: Two QTLs, Cc1-1ian and Cc1-2ian were located on linkage group 1 of clone IAN 873. A: The locations of both QTLs were identified using the Interval Mapping analysis. B: The locations of both QTLs were identified using the multiple-QTL model (MQM) analysis.

and marker P2E2M13192 for *Cc1-2ian* (Appendix - *Fig. 1B).*

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PHILIPPINES

University of Southern Mindanao, Kabacan, Cotabato

University of Southern Mindanao is currently engaged in 2 Biotech Projects for rubber and are as follows:

Development of Molecular Marker for Identification and authentication of *Hevea brasiliensis* Clones

Emma K. Sales and Nilda G. Butardo

1. Selection and Identification of specific SSR Marker for a specific clone.
2. Development of reference standard alleles for rubber.
3. Validation of SSR primers.
   - This project had evaluated morphologically 109 rubber clones from the University of Southern Mindanao germplasm collection. For molecular analysis, 41 SSR primers were used to determine and develop a marker kit for identification and authentication of the rubber clones.

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– A standard or reference allele is being developed out of 41 markers. 22 SSR primers were found to be useful. Validation is still ongoing. Development of a rubber profile is also ongoing.

Development of Simple and Efficient Tissue Culture Technique for Rapid Propagation in Rubber

Romulo L. Cena and Harem Roca

a) Optimization of somatic embryogenesis protocol using different explant sources and 2,4D treatment

b) Exploration of an efficient technique to increase callus formation, reduce browning and reduce contamination. The researcher is experiencing problem on this: hence this activity is relentless being pursued. So far other options have to be tried/tested for a successful friable callus formation.

THAILAND

Rubber Research Institute of Thailand (RRIT) Biotechnology Group

Molecular Biology

Expression of Disease Resistance Genes in Rubber Tree (*Hevea brasiliensis*) Infected by *Phytophthora palmivora*

Phytophthora palmivora is the causative agent of leaf fall and black stripe in rubber plant, *Hevea brasiliensis*. It infects petioles, causing leaves to fall prematurely and attacks the tapping surface resulting in the poor latex production. The expression of genes of two clones (resistance; BPM 24 and susceptible; RRIM 600) against zoospores from *P. palmivora* were investigated comparing with control (BPM 24 and RRIM 600 against sterile distilled water) by using cDNA-AFLP technique which reveals the difference of DNA fragments after polyacrylamide gel electrophoresis.

In comparison with control, the expression of most genes in infected RRIM 600 and BPM 24 are up regulating. It is suggested that these genes are candidate genes involved in disease resistance against *P. palmivora* in rubber tree. These candidate genes from BPM 24 were sequenced and blasted in GenBank and Protein Database (NCBI). The blast result showed that most of them are genes involved in plant defense mechanism, which are involve in peroxidase and phenylalanene ammonia lyase enzymes regulation, and pathogenesis related-protein (ß-1,3 -glucanase chitinase) synthesis and some of them are unknown which may be novel genes. Moreover, semi-quantitative RT-PCR (sqRT-PCR) technique was used to verify if infected time of resistance clone (BPM 24) had a relationship to peroxidase mRNAexpression. In this study, peroxidase mRNA showed a highest expression after the leaves were infected for 18 hour. Peroxidase mRNA may involve in scopolitin (phytoalexin) synthesis in order to defense the cell from pathogen (Churngchow and Rattarasarn, 2001). An expression of this geneand scopolitin content should be examined to verify if these parameters have a relationship to disease resistance level in rubber tree.

Molecular genetic

Association mapping for yield, girth growth and wood property in rubber tree (*Hevea brasiliensis*)

This project is currently being undertaken started from October 2010. The objective of this study is to analyze and identify the association of simple sequence repeat (SSR) markers with yield, girth growth and wood property traits in a panel of one hun-

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dred accessions from the RRI-CH-35 clones and Asiatic clones. Seven Asiatic clones consisted of BPM24, RRIM600, PB260, RRIT251, RRIC100, RRIC 110 and RRIC111. The RRI-CH-35 clones resulted from controlled crossings and self pollinations performed in Chaseongsoa Rubber Research Center (CRRC) Thailand. Recently, fifty SSR markers were evaluated for genotypic analysis.

Exploiting EST databases for the development and characterization of EST-SSR markers in rubber tree (*Hevea brasiliensis*)

In total, 10,321 full-length cDNA from SSH library which developed by rubber biotechnology research group, Mahidol university Thailand, were used to evaluate for EST-SSR. Four hundred thirty one SSR sites were identified by data mining of which 291 EST sequences were found. The EST-SSR markers will use for QTL mapping in population RRIM600 X PB217.

VIETNAM

RUBBER RESEARCH INSTITUTE OF VIETNAM

Molecular analyses of genetic variability in *Hevea*

The strategy of extending rubber tree to non-traditional regions in Vietnam necessitates breeding of rubber clones tolerant to environmental constraints of any particular region. In order to obtain a large quantity of *Hevea* clones adapting to different ecological regions, the breeding materials need to be genetically diverse since the selection of advanced materials could be more effective for population harboring large genetic variability. For more effective integrating genetic resources into breeding program suitable for the above-mentioned strategy, a study was conducted using the Random amplified polymorphic DNA (RAPD) technique to investigate the genetic diversity and structure of *Hevea* germplasm in Vietnam. A total of 250 genotypes representing for 17 districts of Amazonian resource (A) and 7 sub-groups of oriented-selected Wickham (W) and Wickham-Amazon (WA) resources and 8 arbitrary primers were brought into the study. A total of 137 DNA fragments were amplified by 8 primers from the investigated population. Analyses showed that there was a high level of genetic variability in this germplasm as demonstrated by a high percentage of polymorphic band (98.54%); a high value of mean heterozygosity (0.1932-0.2390); a high value of Shannon's diversity index (0.3731-0.4024) and a large genetic distance among genotypes (pairwise Nei's genetic distance values from 0.075 to 0.731). The A genetic resource was more diverse than the W resource. The total molecular variance of the W resource approximated to 75.89-83.42% of that of the A resource and approximated to 82.79% of that of the WA resource. Therefore, the narrow genetic variability of the W resource can be improved by hybridizing orientedly with Amazonian genotypes. The molecular variance analysis (AMOVA) revealed that most of the genetic variations (85.47%) were found among genotypes within the districts or sub-groups, while this variance component for inter-distrists/-sub-groups and -resources accounted for only 9.09% and 5.39% respectively. Dendrogram showed various degrees of separation among the districts/sub-groups and the relationship among genotypes originated from 17 geographical origins of Amazonian resources and from 7 sub-groups of Wickham and Wickham-Amazon resources. The dendrogram indicated that Wickham resource was genetically closer to the genotypes from the state of Mato Grosso than those from the Acre or Rondonia. The results

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from this study showed that *Hevea* germplasm in Vietnam is very diverse and abundant, which will be a valuable genetic resource for breeding program.

In another study, the same set of 8 primers and RAPD technique was used to evaluate genetic variability of the *Hevea* hybrids derived from five crosses of maternal clone PB260 (Wickham-W) with five fatherly Amazonian (A) genotypes. A total of 101 DNA fragments of 180-2300 bp were amplified from 147 hybrids and 6 parental genotypes. Regarding to each cross, the percentage of polymorphic DNA bands varied from 67.19 to 94.87; the pairwise Nei's genetic distance values ranged from 0.025-0.706; the mean heterozygosity varied from 0.127 to 0.195; and the estimated Shannon's diversity index ranged from 0.300 to 0.408. In comparison with parental DNA banding patterns, the source of genetic variations among hybrids revealed to be the combinations of genetic differences of their parents. Molecular variance analysis indicated that 62% of genetic variations were within individuals in crosses while the rest of 38% were found among crosses, suggesting the importance of enlarging different crosses for enhancing genetic variability in progenies. The correlation analysis between parental genetic distance and corresponding hybrid's genetic variability suggested the possibility of increasing genetic variation in *Hevea* progenies through selecting parents. UPGMA cluster analysis segregated the studied hybrids into four distinct groups irrespective of crosses. The study showed that genetic variability in the W × A hybrids was rather high and can be enhanced by crossing between two far genetic-distance parents.

These studies provided useful information for appropriate utilization of *Hevea* genetic resource into breeding program such as crossing between parents having far genetic distance for exploiting heterosis and the possibility of combining suitable genetic resources for both improving genetic variability and agronomic traits in commercial rubber clones. The high genetic variability of the W × A hybrids, as expected, provided a great source of materials for selecting advanced *Hevea* clones adapting to adverse environment.

Molecular studies on *Hevea* diseases

Molecular analysis of *Corynespora cassiicola* Isolates from Rubber Tree and Some Other Hosts in Vietnam Using rDNA ITS Sequencing and ISSR Markers

Thirty eight (38) *Corynespora cassiicola* isolates collected from different clones of rubber tree and some other hosts namely papaya *(Carica Papaya), Ecdysanthera rosea,* wild mango *(Irvingia malayana),* white pumpkin *(Benincasa cerifera)* and sesame *(Sesamum indicum)* in Vietnam was analysed using Ribosomal DNA ITS (rDNA ITS) sequencing and Inter Simple Sequence Repeat (ISSR) markers. In this study, rDNA ITS sequences segregated the 38 studied isolates into two distinct groups based on the Single Nucleotide Polymorphisms (SNP) detected at base pair 135 in ITS1 region. Group 1, includes 21 isolates, in which 19 isolates collected from rubber tree at Lai Khe, Dau Tieng, Dong Phu, Dong Nai, Quang Nam and 2 isolates collected from papaya at Cu Chi, contained Cytosine (C); and group 2, includes 17 isolates in which 11 isolates from rubber tree at Lai Khe, Dau Tieng, Tay Ninh, Phu Giao, Dong Phu, Dong Nai, Quang Nam, Highland; 1 isolate from sesame at Dau Tieng 2 isolates from papaya at Lai Khe and Dong Phu; and 3 isolates from *Ecdysanthera rosea,* wild mango and white pumpkin at Lai Khe contained Thymin (T). Meanwhile, the ISSR markers analyses with 8 primers have produced 88 DNA bands in which 94.3% were polymorphism. Dendrogram produced from

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UPGMA analysis based on Nei and Li's coefficient also divided the 38 studied isolates into 2 main clusters. The isolates in each cluster corresponded to those of 2 groups obtained from rDNA ITS sequencing analyses. The present of both 2 groups of isolates was detected in almost areas suggested maybe the pathogen has been moving from regions to regions. In addition the cluster 1 was separated into 2 subcluster in which the subcluster 1B only contained isolates from Lai Khe including 6 isolates from rubber tree and cluster 2 was also separated in to 2 subcluster in which the subcluster 2B also only contained isolates from Lai Khe consist of 1 isolate from rubber tree and 4 isolates from other hosts (papaya, white pumpkin, wild mango and *Ecdysanthera rosea*). These results inferred close genetic relations among some isolates at the same area and there are some distances among isolates obtained from different areas.

Molecular studies for *Hevea* clone identification

The narrow genetic base of the cultivated *Hevea* clones together with the closely related pedigree of the present-day commercial clones has made it difficult for morphological identification of current *Hevea* commercial clones. For more effective management of *Hevea* clones in the budwood nursery and experimental system, the application of Inter Simple Sequence Repeat (ISSR) markers for Hevea clone identification have been developed at the RRIV. Twenty ISSR primers were screened to select six 3'-anchored ISSR primers that yielded a highest level of polymorphism and the reliable and reproducible banding patterns. These ISSR primers were used to amplify DNA samples extracted from ten commercial clones that have been currently recommended in the class I and class II (55% and 40% of total planting area, respectively) of rubber planting recommendation 2006-2010. A total of 48 DNA fragments ranging from 240-1600 bp were amplified and 25 fragments were polymorphic in these ten *Hevea* clones. The study showed that only two primers namely T30 and T34 ($(TCC)_5C$ and $(GA)_8A$) could be used to distinguish any clone among the ten studied *Hevea* clones, while the set of six under-studied primers revealed to be useful for identification any of these clones. The study revealed the applicability of these ISSR markers for clone identification and also for analysis of genetic relationship among commercial *Hevea* clones. This currently developed technique will be beneficial for better management of *Hevea* clones in the source-bush nursery and experimental system.

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International Rubber Research and Development Board

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Secretariat: 260, Jalan Ampang, 50450 Kuala Lumpur

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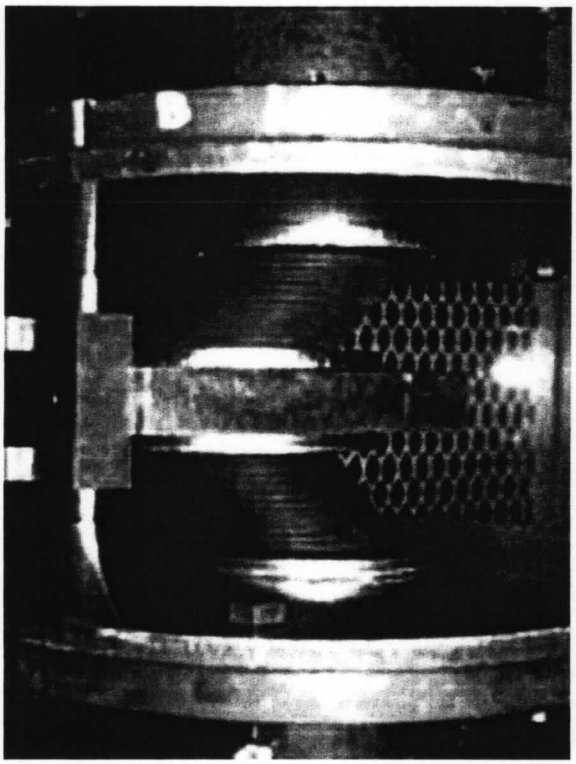

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